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Detection of Salmonella Typhi in Urine of 6-12 Years Old Children in the Coastal Region of Southeast Sulawesi Using the Polymerase Chain Reaction Method

Salfitriana Rebecha Lakawa[a], Wa Ode Salma[b*], Yenti Purnamasari[c], Asriati[d], Irawaty[e], Adius kusnan[f], Sri Susanty[g], Rahmawati[h]

[a]*Medical Study Program, Faculty of Medicine, Halu Oleo University, Kendari Indonesia*

[b]*Department of Nutrition, Faculty of Medicine, Halu Oleo University, Kendari Indonesia*

[c]*Microbiology Laboratory of Faculty of Medicine, Halu Oleo University, Kendari Indonesia*

[d]*Department of Epidemiology, Faculty of Medicine, Halu Oleo University, Kendari Indonesia*

[e]*Department of Clinical Pathology, Faculty of Medicine,, Halu Oleo University, Kendari Indonesia*

[f,g,h]*Department of Nursing, Faculty of Medicine, Halu Oleo University, Kendari Indonesia*

[a]*Email: salfitrianarebechalakawa@gmail.com ;* [b*]*Email: waode.salma.fkuho@gmail.com*

[c]*Email: yenti.purnamasari@ymail.com ;* [d]*Email: asriatiyusuf@gmail.com ;* [e]*Email: bigfatbabyhuey@gmail.com*

[f]*Email: adius_kusnan@yahoo.com;* [g]*Email: srisusantysahir@gmail.com;* [h]*Email:rahmawati.kdi82@gmail.com*

Abstract

Typhoid fever caused by Salmonella typhi bacteria still become a global health problem throughout the world, especially in developing countries. Previous study reports showed that Salmonella typhi is one of the causes of urinary tract infections in children which suspected of lack of access to clean water, contaminated food and poor environmental sanitation. This study aimed to detect Salmonella typhi bacteria in the urine of children at aged 6-12 years, residing in the Coastal Region of Southeast Sulawesi Province, Indonesia. Measuring instrument to detect Salmonella typhi bacteria in 50 mid-stream urine while in children using the Polymerase Chain Reaction (PCR) method with amplification product length was 302 bp. As much as 47 samples of Nutrient Broth (NB) were positive (94%) and only 3 (6%) samples gave a negative result. The results of the study showed that from 94% of the urine samples of children, Salmonella typhi was detected in their urine as much as 24% with a distribution of 18% female and 6% male.

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* Corresponding author.

Keywords: *Salmonella typhi*; children, Urine; PCR.

1. Introduction

Salmonella typhi (S.typhi) is bacteria the cause typhoid fever and it has high mortality and morbidity throughout the world [1]. Global problems related to typhoid fever are estimated to reach 12 million cases and 130,000 deaths in 2010 due to typhoid fever. Previous studies reported that S. typhi bacteria is one of the causes of urinary tract infections in children, they have been associated with the incidence of structural abnormalities or immunosuppression status and are associated with the incidence of urolithiasis [2].

Indonesia is one of the tropical countries and referred to 5 countries in Asia that are endemic to typhoid fever, especially infected in children and associated with poor sanitation and hygiene, or food and beverage intake that contaminated by S. typhi [3,4,5]. The incidence of typhoid fever in Indonesia in 2008 amounted to 81.7 per 100,000 population with the highest age group was aged 2-15 years with the spread of events in children aged 2-4 years amounting to 148.7 / 100,000, and in children aged 5-15 year amounting to 180.3 / 100,000 inhabitants [6]. While the incidence of typhoid fever in Southeast Sulawesi is still very high with the number of cases amounted to 1,867 cases in 2015 [7] and increased to 4,641 cases in 2016 [8].

Less rapid and less accurate diagnosis supporting test can cause a high mortality rate for typhoid fever. Currently serological tests such as the Widal test were the method that used in diagnosing typhoid fever, but the sensitivity and specificity of the Widal test are still low because the test did not have a positive test standard [9]. The Hatta and Smits (2007) study report stated that the detection of S. typhi was carried out using the PCR method. It was found that the sensitivity and specificity were much higher compared to only serological tests such as the Widal test. In addition, PCR was able to detect the amount of pathogenic DNA through amplification of DNA segments and was able to distinguish other organisms [10].

There was an increase in the incidence of typhoid fever in Southeast Sulawesi Province, so we are interested and wanted to conduct studies with the aim of detecting S. typhi in the urine of elementary school students who reside in the Coastal Region using Polymerase Chain Reaction measuring instruments.

2. Materials and Method

2.1. Group Subject

The sample in this study amounted to 50 urine samples of children aged 6-12 years. Sampling is carried out from November to December 2018, at 04 Abeli Public Elementary School, Kendari City, Southeast Sulawesi, Indonesia. To detect S. typhi in the urine of children, it was conducted at the Microbiology Laboratory and Laboratory of Research and Biomolecular, Faculty of Medicine, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia. This research was approved by the Health Research Ethics Commission of Halu Oleo University, Kendari, Southeast Sulawesi, with recommendations for ethical clearance number: 2341 / UN29.20 / PPM / 2018.

2.2. Procedure for urine sampling

The urine sample that takes was divided into 2 sessions, for the first session urine was taken from students in grades 4-6 and continued with second session by the next day in grades 1-3 students with a new urine container. The sample then placed in an ice box as a temporary shelter from the sampling location to the UHO Medical Faculty Laboratory.

2.3. Samples Inoculation in Nutrient Broth (NB)

Inoculation was done at the Microbiology Laboratory of FK UHO by dissolved 4.8 grams of NB in 600 ml of aquadest, then it was boiled by a hot plate and homogenized by using a magnetic stirrer. Then it was sterilized with autoclave at 121 ℃ for 15 minutes. Samples then inoculated in NB according to the method reported by Himedia [11], using sample storage media in the form of NB medium. Each child's urine sample was inoculated into a medium in the form of nutrient broth, so that a total of 50 NB was obtained containing urine samples, then incubated for 24-48 hours at 37 ℃. The results of the observations was observed after 48 hours, obtained as many as 47 positive NB samples and 3 samples gave a negative result. Furthermore, Bacterial identification techniques will be carried out in 47 positive NB samples through the stages of bacterial DNA extraction and measurements using the PCR method.

2.4. DNA extraction

DNA extraction was carried out according to the procedure of the Wizard® Genomic DNA Purification kit [12], in short as follows: as much as 1.5 ml of urine culture in NB which was incubated for 48 hours was put into a 1.5 mL eppendorf tube, then centrifuged at 13,000 rpm for 2 minutes and then the supernatant was removed. Then add 600 µl of nuclei lysis solution and then vortex then it was incubated at 800C for 5 minutes and cooled at room temperature. Add 3 mL of RNAase solution to the cell lysate to degrade RNA so the only thing remains was DNA. The tube was tossed and turned 2-5 times to mix, then it was incubated 37^0C for 15 minutes and cooled at room temperature. Add 200 µl of solusen precipitation protein solution to precipitate the protein, then vortex at high speed for 20 minutes then it was incubated 5 minutes, then it was centrifugated at 13,000 rpm for 5 minutes. Next, moved the supernatant that contained DNA to the new eppendorf that contained 600 µl of isopropanol to remove the residue from the extraction stage. Then mix well until you see strands of yarn which are DNA mass, then centrifuged at 13,000 rpm for 2 minutes. Then carefully pour the liquid on the eppendorf onto the absorbent paper and add 600 µl of 70% ethanol to purify the DNA, tossed and turned the tube slowly, then centrifuged at 13,000 rpm for 2 minutes, discarding ethanol. After that, centrifuged and remove the liquid on the filter paper and allow the pellet to dry for 15 minutes and add 100 µl of the rehydrated DNA solution to the tube to dry or evaporate and then incubated at 2-80C. This process produces extracts of S. typhi DNA bacterial. The extraction results are then stored for 24 hours at -20 ℃.

2.5. Polymerase Chain Reaction Assay

Polymerase Chain Reaction amplification was carried out in several stages, using a reaction volume of 12.5 µl Gotaq® Green Master Mix PCR system that consisted of; 6.25 µl Gotaq Green Master mix, 1.25 µl of primer 10

µM Forward, 1.25 µl Reverse 10 µM Primer, 2.5 µl DNA sample and 1.25 µl of free nuclease water. Amplification of S. typhi begins with initiation (predenaturation) of 1 cycle at 95^{o}C for 25 seconds, then continue as many as 30 cycles of denaturation at 94^{o}C for 30 seconds, annealing 55^{o}C for 1 minute and extension 72^{o}C for 30 seconds and ending with final extension 72^{o}C for 7 minutes for 1 cycle. The nucleotide sequence of the primary pair used for S. typhi bacteria is HSP701 in sequence (5'-GGA TCC, GAC, GCA, GG, TGT, GAT, GTG, AA-3 ') (forward) and 5'– AAG CTT AAA. GTA CCA CCA CCA CC '(reversed) with the location of locus attachment was STY0012 11891-12192, and it was produced an 302 base pairs amplicon [13]. Furthermore, to see the results of amplification on PCR, an electrophoresis process was performed on PCR products on 100 volt agarose for 30 minutes, then read on ultraviolet light.

3. Results

Table 1. Shows the results that of the 94% of children who were tested for urine using the PCR method it was found that 24% of their urine samples contained S. typhi bacteria with a distribution of 18% female and 6% male.

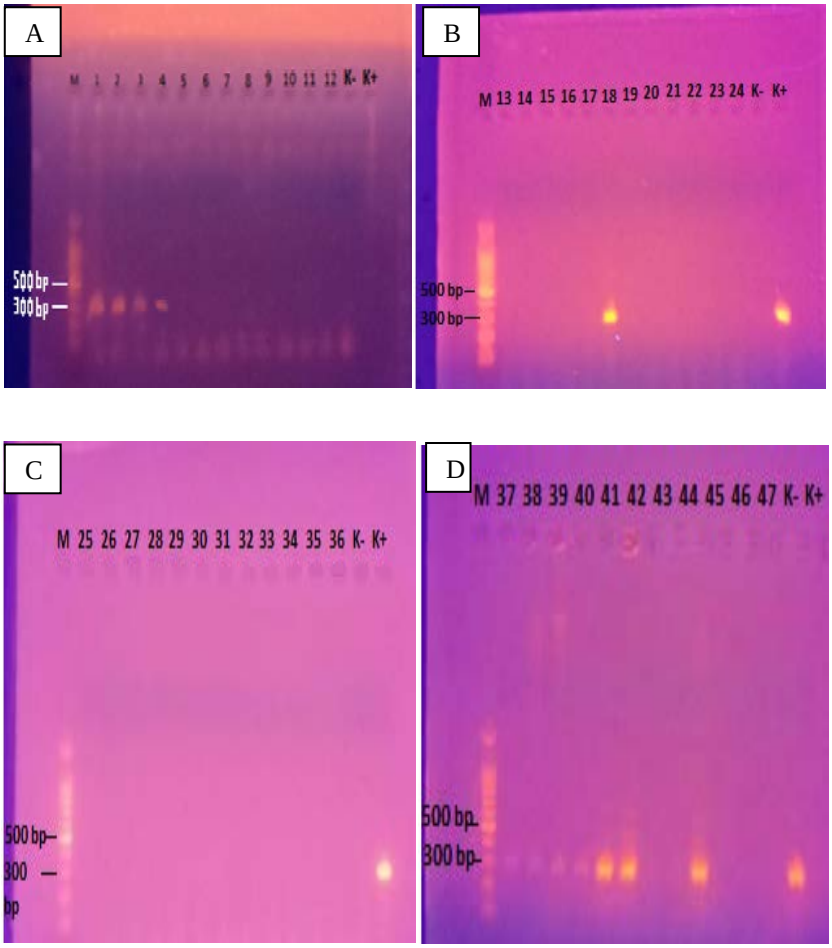

Figure 1: PCR Results of Urine Isolates from Children of 04 Abeli Elementary School. (A) PCR results of samples 1-12, (B) PCR results of samples 13-24, (C) PCR results of samples 25-36, (D) PCR results of samples 37-47, all results are read by electrophoresis method with product length 302 bp**.**

Table 1: Results of Urine Polymerase Chain Reaction Samples Based on Gender from Children of 04 Abeli Elementary School

No.	Gender	NB result	*S. Typhi* PCR			
			Positive		Negative	
			n	(%)	n	(%)
1.	Female	24	9	18	15	30
2.	Male	23	3	6	20	40
	Total	47 (94%)	12	24	35	70

4. Discussion

S. typhi is a gram negative bacteria that causes typhoid fever which is transmitted generally through food and can also through contaminated drinking water due to poor environmental sanitation or due to poor hygiene in individuals [14], often occurred in children aged 2- 15 years old [6]. Another study by Britto and his colleagues explained that the prevalence of typhoid fever based on age often occured in children aged 5-9 years (45%) and followed by children aged 10-14 years (37%) [15]. The study conducted by Akullian and his colleagues reported that age distribution for children and adolescents is known to be positive for children aged 7-11 years in S. typhi in their urine, besides that the morbidity and mortality of typhoid fever is very high in children, possibly because children have natural immunity that is still lacking and has a higher risk of exposure to faecal pathogens [16]. Our study shows that of the 94% of children aged 6-12 years who were tested for urine by the PCR method, it was found that 24% of their urine samples were detected contained asymptomatic S. typhi bacteria. Although this figure is small from data reported from WHO in 2003 that about 91% of typhoid cases occur in children with ages 3-19 years in Indonesia [17]. However, it is very important to prevent and reduce the incidence of S. typhi infection, especially in children.

S. typhi infection has a wide variety of clinical symptoms, ranging from asymptomatic to symptomatic and even life-threatening infections. Our study found positive results of S. typhi in the urine of children but did not provide clinical symptoms of typhoid fever and there was no prior history of typhoid fever. This may be a variation of the clinical symptoms of typhoid fever between individuals that was not same or maybe the number of bacteria in the body was not enough to cause clinical symptoms. The conclusion of the study by Nelwan states that children exposed to S. typhi for a long time can be said to have entered the chronic phase. In the chronic phase, typhoid fever usually does not provide clinical symptoms which means that S. typhi will be detected in individuals through urine [18], will occupy the gall bladder and the individual can be a source of transmission of S. typhi. The same study reported by Gun and his colleagues shows that it is very difficult to detect individuals who have reached the chronic phase of S. typhi infection especially in endemic areas, this is because individuals generally do not have any symptoms, and more than 25% do not has a history of typhoid fever. Another factor that can cause S. typhi detected in children's urine but does not provide clinical symptoms was that there was not enough bacteria in the body to cause clinical symptoms [19]. An infectious dose that can

cause clinical symptoms of typhoid fever is estimated to be 10^6 organisms [20]. In our study, only conventional PCR was examined so that it was not known how many S. typhi organisms were estimated in children's urine. Future research should be carried out using the quantitative PCR method because it has the best quality level and can be known to the number of S. typhi organisms so that estimates for clinical diagnosis will be even better to prevent further exposure.

Salmonella infection which reported based on sex by Reller and his colleagues there was an increase in the incidence of typhoid fever and consistently showed that the infection was more common in women [21]. Epidemiological studies conducted by Yoo and his colleagues Founded that the incidence of typhoid fever in women was higher than in men (women 0.40 per 100,000 population, and men 0.34) [22]. Women have a higher risk of exposure to S. typhi because they may contact with contaminated household appliances. In addition, the anatomical structure of a woman's urinary tract also plays an important role. Women have a shorter urethra and it is closer to the anus, thus facilitating ascending bacterial infection [23]. Interestingly, our study showed that of the 94% of children who were tested for urine using the PCR method it was found that 24% of their urine samples contained S. typhi bacteria with a distribution of 18% female and 6% male.

Currently sanitation and hygiene are certainly a critical global health problem, especially among children in developing countries because they have a higher risk of enteric infection. Sanitation and hiegene aim to prevent contamination that comes from a bad environment [24]. Very high risk in densely populated populations and lack of sanitation and access to clean and safe water as well as clean food, and household hygiene. Food also plays an important role, it is very good to ensure that the food we consumed is clean and well-cooked [24,6]. Recent evidence shows that reservoirs of environmental infections can also support transmission of disease. As a result of our study, 04 Abeli elementary school was located in a coastal area which is a plain area with population density that can be a risk of transmission of S. typhi. In addition, 04 Abeli elementary school does not have a large sewer, so on the rainy season the water is sometimes inundated and does not flow properly. This condition may be a risk of typhoid fever. Typhoid fever is also associated with environmental factors, including proximity to open sewage and highly contaminated water, shelter in low-lying areas, and rainy seasons [16]. Efforts to improve behavior in environmental hygiene and drinking water management such as by cooking, need to be socialized. In addition, the behavior that can make elementary school-age children avoid infection with S. typhi is by washing their hands properly, using soap and with running water and the school environment is required to have a final trash can and have a hygienic toilet.

5. Conclusion

Changing people's behavior was a challenge and that was not easy but it is very important to prevent the occurrence of typhoid fever, by promote changes in people's behavior to keep the environment clean and strive for good sanitation facilities to minimize the risk of exposure to S. typhi transmission.

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